Article

Use of recombinant human thyroid-stimulating hormone for thyrotropin stimulation test in healthy, hypothyroid and euthyroid sick dogs

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Abstract — Recombinant human thyroid-stimulating hormone (rhTSH) was evaluated for the diagnosis of canine hypothyroidism, using TSH response tests. Phase I stimulation tests were performed in 6 healthy dogs weighing over 20 kg, using 50 and then 100 μg of freshly reconstituted rhTSH administered intravenously. In phase II, the same dogs were stimulated by using 100 μg of rhTSH frozen for 3 months at -20°C. Phase III stimulation tests were performed by using 50 or 100 μg of freshly reconstituted or frozen rhTSH in healthy (n = 14), euthyroid sick (n = 11) and hypothyroid dogs (n = 9).

A dose of 100 μg of rhTSH was judged more appropriate for dogs weighing more than 20 kg. Biological activity of rhTSH after freezing at -20°C for up to 12 weeks was maintained. When stimulated, significant ($P < 0.05$) increases in total thyroxine concentration were observed only in healthy and euthyroid sick dogs. Results of this study show that the rhTSH stimulation test is able to differentiate euthyroidism from hypothyroidism in dogs.

Résumé — Utilisation de la thyréotropine humaine recombinée (TSHhr), lors d'un test de stimulation à la TSH, chez des chiens en santé, atteints de maladies systémiques et hypothyroïdiens. La thyréotropine humaine recombinée (TSHhr) fut évaluée pour le diagnostic de l'hypothyroïdie canine à l'aide de tests de stimulation à la TSH. Phase I : des stimulations intraveineuses ont été effectuées chez 6 chiens en santé de plus de 20 kg utilisant 50 et 100 μg de TSHhr nouvellement reconstituée. Lors de la phase II, ces chiens furent stimulés à l'aide de 100 μg de TSHhr congelée depuis 3 mois à -20 °C. Phase III : des stimulations utilisant 50 ou 100 μg de TSHhr nouvellement reconstituée ou congelée ont été effectués chez des chiens en santé (n = 14), euthyroïdiens atteints d'une maladie systémique (n = 11) et hypothyroïdiens (n = 9).

Une dose de 100 μg de TSHhr a été jugée appropriée chez des chiens de plus de 20 kg. La capacité biologique stimulatrice de la TSHhr suite à la congélation à -20 °C jusqu'à 12 semaines, a été maintenue. Lorsque stimulés, la concentration sérique de thyroxine totale fut significativement augmentée ($P < 0,05$) seulement chez les chiens en santé et ceux euthyroïdiens atteints d'une maladie systémique. Cette étude démontre que l'utilisation du test de stimulation à la TSHhr permet de différencier l'euthyroïdie de l'hypothyroïdie chez le chien.

(Traduit par les auteurs)

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Introduction

Hypothyroidism is considered one of the most frequent canine endocrine disorders (1). Most affected dogs have primary hypothyroidism caused by lymphocytic thyroiditis, idiopathic thyroid atrophy, or, more rarely, neoplastic or traumatic destruction (1,2). The gradual loss of thyroid parenchyma eventually leads to reduced serum concentrations of thyroid hormones. These hormones have a wide variety of metabolic functions. The clinical signs of hypothyroidism are therefore numerous, variable, and nonspecific (1–3). Canine thyroid function is now evaluated mainly with serum level determination of total thyroxine (TT_4), free thyroxine (FT_4), endogenous thyroid-stimulating hormone (cTSH), and, in some cases, thyroglobulin autoantibody (TgAA). Unfortunately, not one

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of those tests, alone or in combination with others, has 100% reliability (4–9). Furthermore, systemic nonthyroidal diseases and drug administration can lower TT_4 and FT_4, and, in some cases, raise cTSH serum concentrations (4–13).

Most investigators still regard the TSH response test as the best single test for evaluating canine thyroid function. This dynamic test has the advantage of better differentiating between a hypothyroid dog and one receiving certain medications or suffering from a nonthyroidal systemic illness (1–2,6,8). This test was previously performed by using a bovine source of TSH (bTSH). An appropriate elevation of the TT_4 concentration after IV injection of bTSH was seen with normal thyroid function. However, the pharmaceutical form of bTSH is no longer commercially available. Allergic reactions to the drug, neutralizing antibody formation after repetitive administrations, and the emergence of a spongiform encephalopathy (Creutzfeldt-Jacob disease) has precluded its use in human medicine (5,10). While a chemical-grade of bTSH can still be obtained, it is not approved for clinical purposes and severe anaphylactic-type reactions have been documented with its use in dogs (7,8).

A synthetic form of TSH consisting of recombinant human thyrotropin (rhTSH) (Genzyme, Cambridge, Maine, USA) has recently been introduced in the pharmaceutical market. This glycoproteic molecule is expressed in a line of Chinese hamster ovary cells and then purified by ion exchanges and dye affinity chromatography (14–16). In humans, rhTSH is used mainly to monitor patients with treated thyroid carcinoma (14,16–20).

In veterinary medicine, rhTSH was first used by Sauvé and Paradis to perform TSH response tests in normal euthyroid beagles (21). These authors concluded that an IV dose of 50 μg of rhTSH and measurement of the TT_4 serum concentrations 4 and 6 h after the injection were associated with an appropriate and optimal response (21). In another study to evaluate the effects of obesity and weight loss on thyroid function, results from TSH response tests using rhTSH supported the presence of euthyroidism in these dogs (22). The lyophilized form of rhTSH must be reconstituted with sterile water before use (16). Recently, results of a study on the effect of storage on reconstituted rhTSH showed no loss of biological activity of rhTSH on the canine thyroid occurred following 4 wk of refrigeration or 8 wk of freezing.

The main purpose of the present study was to evaluate the clinical usefulness of the rhTSH stimulation test in dogs. First, the optimal dosage of rhTSH to be used in larger dogs was determined. Second, the effect of storage on the biological activity of rhTSH was evaluated. Third, the clinical diagnostic capability of rhTSH for stimulation testing was assessed in healthy, euthyroid sick, and hypothyroid dogs and compared with FT_4 measurements.

Materials and methods

The studies were performed at the Small Animal Clinic of the Faculty of Veterinary Medicine, University of Montreal. This research project was in accordance with the Canadian Council on Animal Care and was approved by the Ethics Committee of the Faculty of Veterinary Medicine, University of Montreal.

Dogs

Thirty-four privately owned dogs were divided into 3 groups: Group 1, healthy dogs (n = 14); Group 2, euthyroid dogs with concurrent nonthyroidal disease (euthyroid sick dogs) (n = 11); and Group 3, hypothyroid dogs (n = 9).

Dogs entered in the study had to be $>$ 1 y of age. Pregnant bitches were excluded. Dogs in groups 1 and 3 could not have received any medications other than flea and heartworm prophylaxis and routine vaccination within 2 mo prior to inclusion. Prior drug administration in dogs of group 2 was allowed, except for drugs known to significantly affect thyroid function tests in dogs (glucocorticoids, sulfonamides, phenobarbital, acetyl salicylic acid).

To be included in group 1, dogs had to have no appreciable abnormalities on both a physical examination and a routine biochemical profile and complete blood (cell) count (CBC). In addition, to confirm euthyroidism in those dogs, TT_4 values had to be $\geq$ 15 nmol/L and cTSH serum concentrations had to be $\leq$ 0.6 ng/mL.

Group 2 included dogs that were examined or hospitalized with a well-established systemic illness whose diagnosis was based on history, physical examination, results of laboratory tests, and further testing, depending on the disease. Clinical signs were not to be explained by hypothyroidism in those dogs, and TT_4 and cTSH measurements were not used to enter dogs in this group.

To be included in group 3, dogs had to have clinical signs consistent with hypothyroidism (lethargy, weight gain, dermatological abnormalities), the diagnosis had to be confirmed by finding a TT_4 $<$ 15 nmol/L and TSH serum concentrations $>$ 0.6 ng/mL, and the dogs had to have had a good clinical response to therapy with L-thyroxine within 6 mo. In the event that cTSH values were within the reference interval, dogs could still be included as hypothyroid if their FT_4 and TgAA values were low and positive, respectively.

Thyroid-stimulating hormone response tests

Thyrotropin stimulation tests, using rhTSH (Thyrogen; Genzyme), were performed at least once in all dogs after inclusion in the study. One vial of rhTSH was reconstituted with 1.2 mL of sterile water (0.9 mg/mL). Individual doses of 50 μg and 100 μg of freshly reconstituted rhTSH in 1-mL plastic syringes with rubber caps were stored frozen at -20°C for a maximum of 12 wk. Freshly reconstituted or frozen rhTSH was injected through an IV cephalic catheter. If frozen rhTSH was used, it was thawed at room temperature. Blood samples for determination of TT_4 concentration were taken before (T0h) and at 4 (T4h) and 6 (T6h) h after rhTSH administration. Criteria used to classify dogs as euthyroid included either a post-TSH TT_4 concentration at T4h and/or T6h equal to or exceeding 40 nmol/L or an increment (change) of the post-TSH TT_4 concentration over the T0h level by at least 20 nmol/L. This prospective study was divided into 3 distinct phases:

Phase I: determination of optimal doses — The TSH response tests, using 50 μg of freshly reconstituted rhTSH, were performed in 6 healthy dogs from group 1 weighing more than 20 kg. At least 10 d after the 1st rhTSH response test, a 2nd test

was performed in the same dogs, using 100 μg of freshly reconstituted rhTSH. Phase I was used to determine the more appropriate dose of rhTSH (50 μg versus 100 μg) for stimulating the thyroid of dogs weighing more than 20 kg.

Phase II: Effect of freezing — Three months after phase I, a TSH response test was performed in each of the same 6 dogs, using 100 μg (dose determined from phase I) of rhTSH that has been frozen at -20°C for a period of 12 wk. Phase II was used to determine the effect of freezing on the biological activity of rhTSH.

Phase III: Assessment of clinical usefulness — Response tests, using 50 μg of rhTSH for dogs less than 20 kg and 100 μg for dogs ≥ 20 kg were performed in all 3 groups. The rhTSH used was either freshly reconstituted or frozen for less than 12 weeks (usage based on the result of phase II).

Specimen collection and storage

Blood samples for CBCs, biochemical profiles, and the measurement of serum TT_4, FT_4, and cTSH concentrations were taken by jugular venipuncture. Samples for the collection of serum were kept at room temperature for approximately 20 minutes for clot formation prior to centrifugation. Serum aliquots were frozen at -20°C in plastic tubes until assayed.

Endocrine assays

Total thyroxine was measured by using a commercial radioimmunoassay kit (Coat-A-Count Canine Total T_4; Diagnostic Products, Los Angeles, California, USA). The reference interval for TT_4 of euthyroid dogs was 15 to 45 nmol/L (manufacturer's information). Measurement of cTSH by immunoradiometric assay was performed with a commercially available kit (Coat-A-Count Canine TSH IMRA; Diagnostic Products). The reference interval of cTSH of euthyroid dogs was 0 to 0.6 ng/mL (manufacturer's information). The FT_4 was measured after equilibrium dialysis by using a commercial kit (Nichols Institute Diagnostics, San Juan Capistrano, California, USA). The reference interval for FT_4 of euthyroid dogs established by the laboratory performing the assay was 6 to 42 pmol/L. Canine TgAA assays were performed by using a commercially available enzyme-linked immunoabsorbent assay (ELISA) commercially available kit (Oxford Biomedical Research, Oxford, Michigan, USA). The reference interval for TgAA of euthyroid dogs was < 200%. Serum samples for FT_4 and TSH response test-TT_4 from all dogs were assayed at the end of the study. Analysis of FT_4 and TgAA samples were performed at the Endocrine Section, Animal Health Diagnostic Laboratory, Michigan State University.

Data analysis

Thyroid hormone assays — Free T_4 measurements of healthy, euthyroid sick, and hypothyroid dogs were compared by using fixed effects analysis of variance with normally distributed error terms. The hypothyroid dog group was compared pairwise with the healthy and euthyroid sick dog groups at the Bonferroni adjusted significance level of 2.5%.

Thyroid-stimulating hormone response tests — The effect of dose (50 versus 100 μg rhTSH) (Phase I) and the effect of freezing (fresh versus frozen rhTSH) (Phase II) on the TT_4 concentration 4 and 6 h after stimulation were evaluated by a mixed model, with dog as random effect and with time and dose (Phase I) or freezing (Phase II) and their interaction with time as categorical fixed effects. The effect was tested both globally at the 5% significance level and at the 2 timepoints (T4h and T6h) at the Bonferroni adjusted significance level of 2.5%. Furthermore, within each of the groups, the TT_4 concentration at T4h and T6h was compared with the TT_4 concentration at T0h at the Bonferroni adjusted significance level of 2.5%.

The 3 groups (healthy, euthyroid sick, and hypothyroid dogs) were compared with each other by a mixed model, with dog as random effect and time, group, and their interaction as categorical fixed effects. The hypothyroid dogs' group was compared pairwise with the healthy and euthyroid sick dogs' group at T0h, T4h, and T6h at the Bonferroni adjusted significance level of 0.83%. Furthermore, within each of the 3 groups, the TT_4 concentration at T4h and T6h was compared with the TT_4 concentration at T0h, and the concentration at T4h with the concentration at T6h, at the Bonferroni adjusted significance level of 1.66%.

Results

Group 1: Healthy dogs

The healthy dog group was composed of 14 hospital staff-owned dogs ranging from 2 to 12 y (mean 5.5, standard deviation [*s*] = 3.0 y) and weighing between 13 and 46 kg (29.6, *s* = 10.7 kg). They consisted of 7 males (all neutered) and 7 females (1 intact, 6 neutered) and comprised 5 Labrador retrievers, 1 cocker spaniel, 1 Airedale terrier, and 7 mixed breed dogs. Six dogs of this group weighing more than 20 kg participated in phase I and II of the study. They consisted of 5 neutered males and 1 neutered female weighing between 29 and 46 kg (36.8, *s* = 6.5 kg). This subgroup was composed of 3 Labrador retrievers and 3 mixed breed dogs.

Group 2: Euthyroid sick dogs

The euthyroid sick dog group consisted of 11 client-owned dogs ranging from 3 to 11 y (6.5, *s* = 3.0 y) and weighing between 6.8 and 50 kg (24.4, *s* = 17.1 kg). They consisted of 7 males (4 neutered and 3 intact) and 4 females (all neutered) and comprised 1 Labrador retriever, 3 miniature schnauzers, 1 Lhasa apso, 1 Welsh corgi, 1 German shepherd, and 4 mixed breed dogs. The types of nonthyroidal diseases recorded were chronic renal failure (*n* = 2), acute renal failure (*n* = 1), severe pancreatitis (*n* = 1), aspiration bronchopneumonia (*n* = 1), lymphoma (*n* = 2), nonlymphoid neoplasia (*n* = 2), diabetes mellitus (*n* = 1), and extrahepatic biliary obstruction (*n* = 1). Clinical signs, biochemical profiles, and CBCs were compatible with individual systemic illnesses.

Group 3: Hypothyroid dogs

The hypothyroid group included 9 client-owned dogs ranging from 3 to 9 y (4.8, *s* = 1.8 y) and weighing between 16.5 and 60 kg (38.1, *s* = 14.5 kg). They consisted of 5 males (3 neutered and 2 intact) and 4 females (all neutered) and comprised 2 golden retrievers, 2 cocker spaniels, 1 Dalmatian, 1 German shepherd, 1 Labrador retriever, and 2 mixed breed dogs. Two

Table 1. Results of baseline thyroid hormone serum concentrations in healthy (group 1), euthyroid sick (group 2) and hypothyroid dogs (group 3). Results are presented with mean ± standard deviation (s) and range (lowest to highest). Only free thyroxine (FT_4) values are compared. Dog groups with the same superscripts do not differ significantly

	TT_4 (nmol/L)	cTSH (ng/mL)	FT_4 (pmol/L)
Group 1	30.41, *s* = 7.63	0.29, *s* = 0.15	29.78, *s* = 10.72[a]
	15.38–40.50	0.06–0.60	16–57
Group 2	16.34, *s* = 6.48	0.13, *s* = 0.05	21.45, *s* = 10.41[a]
	4.10–25.62	0.05–0.23	6–40
Group 3	4.30, *s* = 3.63	3.43, *s* = 2.94	1.55, *s* = 2.12[b]
	1.07–10.80	0.21–8.27	0–5

TT_4 — Total thyroxine
cTSH — Endogenous thyroid-stimulating hormone

hypothyroid dogs had serum cTSH levels within normal limits, 0.21 and 0.55 ng/mL. Both dogs were included in the hypothyroid group because their TT_4 concentrations were extremely low (< 2.10 nmol/L) and because both dogs were TgAA positive and their FT_4 values were very low.

Hypercholesterolemia was detected in 6 of the 9 (67%) hypothyroid dogs and values ranged between 4.69 and 32.10 mmol/L (12.67, *s* = 8.58 mmol/L) (laboratory reference interval: 2.85 to 7.76 mmol/L). Mild nonregenerative anemia was also present in 5 (56%) of these dogs and packed cell volumes ranged between 0.28 and 0.50 L/L (0.376, *s* = 0.063 L/L) (laboratory reference interval: 0.37 to 0.52 L/L). There were no other significant findings on biochemical profiles and CBCs.

Thyroid hormone assays

The initial values for TT_4, FT_4, and cTSH serum concentrations are given in Table 1. We only compared the FT_4 values between the 3 groups as the TT_4 and cTSH concentrations were used to define the euthyroid normal and hypothyroid groups.

The serum FT_4 concentration differed significantly among the 3 groups ($P < 0.001$) with both the healthy dogs ($P < 0.0001$) and the euthyroid sick dogs ($P < 0.001$) having significantly higher concentrations than the hypothyroid dogs. Extremely low levels were observed in hypothyroid dogs compared with those in euthyroid sick and healthy dogs.

Thyroid-stimulating hormone response tests

Phase I — The serum TT_4 concentration was significantly influenced following IV administration of freshly reconstituted rhTSH ($P < 0.001$) and by the dose used ($P = 0.0053$), with no significant interaction ($P = 0.106$) between time from rhTSH administration (4 to 6 h) and dose of rhTSH (Table 2). Serum TT_4 concentration at T4h and T6h was significantly increased compared with that at T0h following administration of 50 μg of rhTSH. After IV administration of 100 μg of rhTSH, there was also a significant increase in serum TT_4 concentration at T4h and T6h compared with that at T0h. At T6h, the IV administration of 100 μg of rhTSH provided a serum TT_4 concentration that was significantly higher ($P = 0.0045$) than that with the administration of 50 μg (Table 2). Only 5 of the 6 dogs of phase I weighing > 20 kg when stimulated with 50 μg of rhTSH met criteria established for euthyroidism, while all dogs were classified as euthyroid when 100 μg of rhTSH was used. Based on these results, 100 μg of rhTSH was chosen for dogs weighing more than 20 kg, while dogs weighing less than 20 kg received 50 μg for TSH stimulation testing in phase II and III of the study.

Phase II — There was a significant difference ($P < 0.001$) between serum TT_4 concentration at T4h and T6h compared with that at T0h following IV administration of 100 μg of fresh or frozen rhTSH (Table 2). Serum TT_4 concentration differed significantly between freshly reconstituted and frozen rhTSH at T4h ($P = 0.009$) but not at T6h ($P = 0.053$) (Table 2). Only 4 of the 6 dogs met the TSH stimulation test criteria established for euthyroidism when using frozen rhTSH.

Phase III — The IV administration of rhTSH significantly ($P < 0.001$) influenced the serum TT_4 concentration and there was also a significant difference among the 3 groups ($P < 0.001$). In the 14 healthy dogs, there was a significant increase ($P < 0.001$) in serum TT_4 concentration at T4h and T6h compared with that at T0h, but no significant difference between T4h and T6h could be demonstrated. Similarly, the 11 euthyroid sick dogs demonstrated a significant increase ($P < 0.001$) in serum TT_4 concentration at T4h and T6h compared with that at T0h, but no significant difference between T4h and T6h could be demonstrated. However, IV administration of rhTSH in 9 hypothyroid dogs did not provide significant increases in serum TT_4 concentration between T0h, T4h, and T6h (Figure 1). At T0h, T4h, and T6h, healthy and euthyroid sick dogs differed significantly from hypothyroid dogs ($P < 0.001$) in terms of serum TT_4 concentration. Three of the euthyroid sick dogs (27%) did not classify as euthyroid according to TSH response test criteria previously mentioned. When comparing baseline serum TT_4 of euthyroid sick dogs with suboptimal response to rhTSH (8.66, *s* = 4.81 nmol/L) with those that adequately responded (19.22, *s* = 4.33 nmol/L), a significant difference ($P < 0.0066$) was observed.

The rhTSH stimulation test in the context of this study adequately identified 22 of the 25 euthyroid dogs and all the hypothyroid dogs.

Even though some dogs received more than 1 IV administration ($n = 6$) of rhTSH, no side effects or anaphylactic reactions were observed.

Discussion

Thyrotropin has a very important role in the homeostasis of thyroid function. Even though TSHs can differ at the molecular and immunologic level among species, they share similar biological activity (1). This explains why canine and human thyroid glands respond to bTSH administration (1,24). More recently, rhTSH has been shown to actively bind to mice and rat thyroid receptors (25). Thyroid stimulation by rhTSH has also been demonstrated in the Rhesus monkey (26). Similar to bTSH, rhTSH administration can cause an elevation of serum canine thyroid hormones (21–23).

Thyrotropin stimulation tests using bTSH have long been considered the gold standard for the diagnosis of canine hypothyroidism (1,2,6–8). Since the commercial withdrawal of medical grade bTSH, several studies have looked at rhTSH as a replacement (21–23). Based on the results of the present study,

Table 2. Results of phase I and II are shown. Serum total thyroxine (TT_4) concentrations at baseline (T0), 4 (T4h), and 6 (T6h) hours after intravenous administration of 50 or 100 μg of freshly reconstituted or frozen recombinant human thyroid-stimulating hormone (rhTSH) in 6 large healthy dogs. Results are presented with mean and standard deviations (*s*) and range (lowest to highest). Fresh and frozen doses at a particular timepoint (in the same column) with the same letter do not differ significantly

Dosage rhTSH used and storage	Baseline TT_4 (nmol/L)	TT_4 4 h post-TSH (T4h)	TT_4 6 h post-TSH (T6h)
50 μg, freshly reconstituted	21.46, *s* = 6.01 11.70–27.21	45.71, *s* = 10.78 33.90–60.99	45.79, *s* = 15.80 22.80–58.96
100 μg, freshly reconstituted	21.63, *s* = 6.45[a] 13.80–32.40	53.92, *s* = 10.34[a] 45.40–72.10	57.82, *s* = 14.56[a] 44.20–85.20
100 μg, frozen	23.78, *s* = 7.01[a] 16.90–34.80	43.41, *s* = 15.23[b] 26.75–70.40	50.23, *s* = 11.84[a] 35.59–65.90

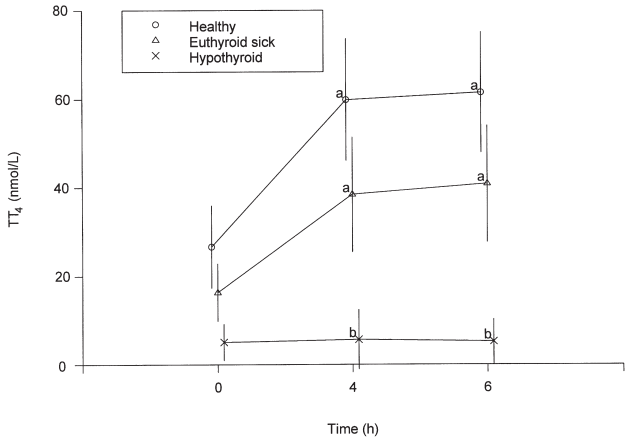

Figure 1. Mean and standard deviation of serum total thyroxine (TT_4) concentrations at baseline and after intravenous administration of 50 or 100 μg of freshly reconstituted or frozen recombinant human thyroid-stimulating hormone (rhTSH) in 14 healthy, 11 euthyroid sick, and 9 hypothyroid dogs. Dog groups with the same letter do not differ significantly.

we conclude that TSH stimulation using rhTSH can differentiate between hypothyroid and euthyroid dogs. Following rhTSH administration, a significant elevation of the serum TT_4 concentration was found in euthyroid healthy (group 1) and sick dogs (group 2) compared with hypothyroid dogs (group 3). Although a significant hormonal rise was observed in group 2 dogs, the response was not as pronounced as in group 1 dogs (Figure 1). In fact, 3 dogs of group 2 were not classified as euthyroid based on criteria established for this study. This agrees with previous work showing that euthyroid dogs with decreased serum TT_4 concentration because of illness or drug administration did respond to bTSH administration, but the response was also often suppressed (1). Severity of illness has been correlated with the degree of serum TT_4 concentration suppression (27). Severe systemic disease can therefore result in a post-TSH serum TT_4 concentration in the interval considered diagnostic for hypothyroidism (1). Euthyroid sick dogs that had suboptimal responses to rhTSH were among the 4 dogs with an initial low baseline serum TT_4 concentration. Baseline serum TT_4 concentrations were also significantly lower for euthyroid sick dogs that failed to classify as euthyroid, compared with other group 2 dogs. Based on those observations, we can hypothesize that the degree of systemic illness in those 3 dogs was greater. The diagnoses in those 3 dogs were pancreatic abscess with severe pancreatitis, ruptured splenic hemangiosarcoma, and stage IVb lymphoma. Rarely would hypothyroidism be primarily suspected and tested for in animals with such debilitating diseases. When used in a more realistic clinical setting, results of rhTSH response testing would probably be even more discriminative. Suboptimal elevation of canine TT_4 serum concentrations after bTSH administration, using a reduced dose protocol, has been documented previously (28). However, the failure of some dogs in group 2 to have adequate response to rhTSH was not a weight-related phenomenon in our study. Indeed, the dose of rhTSH used was 3.43, *s* = 1.40 μg/kg bodyweight (BW) in the studied dogs (*n* = 34), and 3.71, *s* = 1.78 μg/kg BW (*n* = 3) and 4.29, *s* = 1.73 μg/kg BW (*n* = 8) in euthyroid sick dogs that did and did not meet euthyroid criteria, respectively. These doses were statistically similar.

In our study, FT_4 measurement correctly identified all dogs suffering from systemic illnessess as euthyroid. The measurement of FT_4 after equilibrium dialysis is, however, not routinely available in many countries.

The euthyroid sick dogs included in the study had well established systemic illnesses. All observed clinical signs could be attributed to the systemic disease recently diagnosed. None of these dogs had a history prior to development of this systemic disease that could suggest the presence of hypothyroidism. Also, FT_4 measurements were within reference limits for all euthyroid sick dogs. Therefore, concomitant hypothyroidism in these dogs was extremely unlikely.

The doses of rhTSH used in the present study were initially based on previous research, which reported that IV administration of 50 μg caused optimal canine thyroid stimulation (21). In that study, no significant difference was seen between post-rhTSH TT_4 concentrations when doses of 50 μg and 100 μg were compared (21). Since all dogs in the earlier study weighed between 10 and 16 kg, the present study evaluated the difference among serum TT_4 concentration increases after IV administration of 50 μg and 100 μg of rhTSH in heavier dogs (phase I). In phase I of the present study, the post-TSH TT_4 concentrations at 6 h postinjection were higher when 100 μg of rhTSH was used. Also, 1 healthy dog did not meet the criteria used to establish euthyroidism with the rhTSH stimulation test when

using 50 μg, compared with none when using 100 μg. A higher dose was therefore judged more appropriate for dogs weighing more than 20 kg.

Storage of bTSH has been studied previously (29). Biological activity of reconstituted bTSH stored at 4°C was maintained for at least 3 wk. Two other studies showed that bTSH can be stored at -20°C without loss of significant thyroid responsiveness for at least 3 mo and for 200 d, respectively (30,31). Although the manufacturer states that a reconstituted solution of rhTSH can not be stored for more than 24 h at temperatures between 2°C and 8°C, results of a recent study indicated that rhTSH maintained adequate biological activity for at least 4 wk at 4°C, and 8 wk at -20°C (23).

In this study, biological activity of rhTSH was maintained after 12 wk of freezing, although the magnitude of TT_4 increase after rhTSH administration was less optimal with the frozen then with freshly reconstituted rhTSH. Indeed, in the present study, 2 dogs in phase II failed to meet established euthyroid criteria. Reasons for this observation are not clear. Underlying subclinical hypothyroidism seems unlikely, since these 2 dogs initially demonstrated euthyroidism when stimulated with newly reconstituted rhTSH. Neither of these dogs showed clinical signs or laboratory changes (baseline TT_4, FT_4, cTSH, cholesterol concentrations, red blood cell count) suggestive of hypothyroidism. When comparing results of TSH stimulation, using freshly reconstituted rhTSH, for these 2 dogs and the 4 other dogs of phase I, we did not observe a significant difference between TT_4 concentrations at T0h, T4h, and T6h. It is interesting to mention that the dog that did not meet euthyroid criteria in phase I using 50 μg of rhTSH, also did not meet the euthyroid criteria in phase II of the study.

The ability to freeze aliquots of rhTSH and use more than one dose per vial make it more affordable for clinical usage. One aliquot of rhTSH has a similar price to the combined measurement of TT_4 and cTSH.

Storage of rhTSH seems an improbable explanation for the suboptimal rhTSH responses seen in 3 of the 11 euthyroid sick dogs of phase III. Recombinant human TSH used in these 3 dogs was frozen for not more than 5 wk, and 1 dog received newly reconstituted rhTSH. Other euthyroid sick dogs were administered rhTSH that had been frozen for up to 11 wk without evidence of decreased ability to induce an elevation of TT_4.

In both groups of euthyroid dogs (groups 1 and 2), the mean post-TSH TT_4 concentration observed at T6h was somewhat higher than at T4h, although statistical significance was not present. A similar observation was made in previous studies (21–23). The time at which the post-TSH serum TT_4 concentration maximally increases is dependent on the dosage of exogenous TSH (1). Peak post-TSH TT_4 concentration sampling time using a conventional dose (0.1 U/kg BW) of bTSH is reported to be between 4 and 8 h (1,21,28,31). Historically, most laboratories used a 6-hour post-TSH sampling time when performing bTSH stimulation (1,5,7,8). However, previous studies showed that the magnitude of increase in TT_4 was somewhat higher at T6h compared with T4h. Therefore, while sampling after 4 h might be more expeditious and is adequate, sampling after 6 h is optimal.

In the present study, we used 1.2 mL of sterile water to reconstitute the lyophilized rhTSH. We recommend that a larger amount of sterile water (such as 6 mL) be used. This will allow larger aliquots of rhTSH to be obtained, which are easier to handle. Also, in this study, after reconstitution, the content of the vial was aspirated with a 22-G needle, this led to a significant loss of rhTSH. Therefore, we recommend, for example, using insulin syringes of 100 units with red rubber caps.

In phase I, a washout period of at least 10 d was allowed between rhTSH stimulation tests. This washout period was appropriate, as previous studies have shown that TT_4 serum concentrations after rhTSH administration peaked after 4 to 6 h, declining thereafter (21).

In contrast to the administration of bTSH, the administration of rhTSH appears to be safe in humans. Only a few adverse effects, such as nausea and headaches, have been reported and none of the patients studied have had detectable serum rhTSH antibodies (16,19). Although rhTSH antibodies were not evaluated in the present study, none of the dogs experienced anaphylactic reactions or evidence of resistance to rhTSH, even after repetitive administration. This agrees with previous studies in which IV administration of rhTSH was not associated with adverse reaction in dogs (21–23).

Further studies on a larger number of dogs are necessary to evaluate the sensitivity and specificity of the rhTSH stimulation test. Again, the specificity is expected to be higher when the rhTSH stimulation test is performed in a clinical setting in dogs suspected of hypothyroidism instead of in systemically sick dogs. Specificity for cTSH measurement in conjunction with T_4 concentrations in most reports is between 69% and 100% (4,5,10,32,33). In 3 of those studies, TSH stimulation, using bTSH, was used to identify dogs with hypothyroidism, and comparison with results of cTSH and T_4 concentrations was made based on this gold standard. In our study, hypothyroidism was excluded if post-TSH stimulation TT_4 concentration was greater than 40 nmol/L and/or if an absolute increase of 20 nmol/L was found at T4h and/or T6h. These criteria were based on previous work performed in healthy beagle dogs and on clinical experience with the rhTSH stimulation test, where the precise criteria used to classify dogs as euthyroid were either a post-TSH TT_4 concentration at T4h and/or T6h equal to or exceeding 45 nmol/L or an increment (change) of the post-TSH TT_4 concentration over baseline of at least 24 nmol/L (21). Clinical use of these criteria in the past made us suspect that they were too stringent. Further, in a recent study, we observed that a few healthy dogs did not meet those initial criteria for euthyroidism. Therefore, we decided to use slightly less rigid criteria than those of Sauvé and Paradis. In 3 previous studies, using bTSH, diagnosis of hypothyroidism was excluded if the post-TSH stimulation TT_4 concentration was higher than 20, 23, and 32 nmol/L, respectively (5,10,32). We preferred to use a more stringent post-TSH TT_4 concentration cut-off value (40 nmol/L), to override the grey zone that might exist between 19 and 38 nmol/L (2,10).

Two limiting factors for the use of rhTSH are 1) the cost and 2) the time required to perform this dynamic test (4 to 6 h). Therefore, determination of a combination of baseline hormones

is easier and more frequently used in practice. The economical storage of rhTSH in aliquots makes the test affordable, providing that the clinic or practitioner invests in buying 2 vials of rhTSH. Thyrotropin response tests, using rhTSH, could be very interesting in a clinical setting for the diagnosis of hypothyroidism in dogs with ambiguous thyroid hormone concentrations (low TT_4 and cTSH within reference interval) or potentially for helping to differentiate primary from secondary hypothyroidism. Further it can also be used in a research setting.

In conclusion, TSH stimulation using rhTSH is a safe, relatively affordable test that can be used in a clinical setting for the diagnosis of canine hypothyroidism. The effect of drug administration on this test has yet to be evaluated. Most importantly, rhTSH stimulation in dogs seems interesting for cases of suspected hypothyroidism that remain elusive with conventional testing, such as with baseline serum total and/or free T_4 and endogenous cTSH concentrations.

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